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14. ABSTRACT
The PDGF family consists of four members, PDGF A, B, C, and D. Human breast carcinoma cell lines show increased PDGFC mRNA expression in cell lines with high tumorigenicity and invasive potential. The typical paradigm of growth factors is extracellular activation of a receptor. However, utilizing cellular fractionation and subcellular localization we also show that PDGFC appears to not only be secreted into the extracellular space, but we also show that PDGFC is also found in the nucleus of breast cancer cell lines. The exact role of nuclear PDGFC is unknown, however, this demonstrates an important shift from the traditional model of growth factors as extracellular effectors to the potential actions of growth factors intracellularly. This may have very important implications clinically. Blocking aberrant growth factors in cancer progression may not be as simple as blocking their actions extracellularly on the receptor. Thus, nuclear localization of growth factors could have important implications in how we understand and target cancer.

15. SUBJECT TERMS
Breast Cancer, Growth Factors, PDGFC

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Introduction

One in eight women in America will be diagnosed with breast cancer in her lifetime (*1*). Excluding skin cancers, breast cancer is the most common cancer diagnosed among American women with 230,480 women expected to be diagnosed with invasive breast cancer in 2011, accounting for 30% of female cancer diagnoses (*1, 2*). In addition, over 57,650 women in the United States will be diagnosed with carcinoma *in situ* of the breast in 2011 (*1, 2*). For women, breast cancer is second only to lung cancer in the number of cancer deaths in the United States (*1*).

A role of Platelet-Derived Growth Factor (PDGF) signaling in breast cancer has been suggested by mounting evidence. Immunohistochemical analysis revealed increased expression/activation of Platelet-Derived Growth Factor Receptor (PDGFR) in advanced human breast tumor tissues (*3-6*). These studies showed localized, membranous PDGFR in periepithelial stromal cell populations in all of the tissues examined, suggesting a paracrine stimulation of adjacent stromal tissue by breast tumor cells (*6*). Moreover, a recent immunohistochemical study of 181 formalin-fixed paraffin-embedded invasive ductal breast carcinomas demonstrated α-PDGFR staining in 39.2% of these tumors (*7*). In addition this α-PDGFR staining showed an association with lymph node metastasis (p=0.0079), Her2/neu expression (p=0.0265), and Bcl2 expression (p=0.0121) (*7*). A significant role for PDGF autocrine signaling in oncogenic growth factor signaling network is also suggested during breast carcinogenesis; PDGF signaling is activated in mammary tumors in MMTV-Neu/TGF-β transgenic mice, and the blockade of autocrine PDGFR signaling with a PDGF-neutralizing antibody was sufficient to block TGF-β-induced epithelial-mesenchymal transition leading instead to apoptosis (*8*). Furthermore, inhibition of PDGFR signaling by the PDGFR inhibitor STI571 (imatinib mesylate, Gleevec®, Novartis) or expression of a dominant negative PDGFR strongly reduced the metastatic potential of these Ras-transformed EpH4 cells in an *in vivo* metastasis assay (*8*).

Platelet-Derived Growth Factors are members of the cysteine-knot growth factor superfamily (as reviewed in (*9*)). The PDGF family consists of four members, PDGF A, B, C, and D (Figure 1). These growth factors form homo- or hetero-dimeric ligands (as reviewed in (*10*)). PDGFs are mitogens for mesenchymal cells such as smooth muscle cells, fibroblasts, and glial cells (as reviewed in (*10-12*)). PDGF receptors are receptor tyrosine kinases. PDGFRs are known to bind homo- or hetero- dimeric PDGF ligands, inducing receptor dimerization and auto-phosphorylation which then leads to signal transduction via several molecules including PI3K, PLC-γ, Raf-1, GAP, and PKC (as reviewed in (*12, 13*)). PDGFRs are expressed in many different cell types including endothelial, epithelial and neural cells and are necessary for embryological development (as reviewed in (*10*)). PDGFC is one of the newest members of the Platelet Derived Growth Factor family. Identified in the year 2000 by three groups who designated it "fallotein" (*14*) and "Spinal Cord-Derived Growth Factor (SCDGF)" (*15*), and finally "PDGFC" (*16*), the PDGFC gene is located on chromosome 4q32 and consists of 6 exons (Figure 2) (*17, 18*). PDGFC and PDGFD are unique from PDGFA and PDGFB in that they contain an N-terminal CUB domain encoded by exons 2 and 3 (*16, 17*) which has been suggested to bind the pericellular matrix and block receptor binding (*19*). Latent homodimers are secreted and then proteolytically activated (*16*). PDGFC

homodimers bind to and activate Platelet-Derived Growth Factor Receptor Alpha homodimers (PDGFR-α/α) (16, 20) or Platelet-Derived Growth Factor Receptor-Alpha/-Beta (PDGFR-α/β) heterodimers (Figure 1) (20, 21). Recently it has been discovered that the PDGFC gene has two mRNA isoforms, full length PDGFC (FL-PDGFC) and truncated PDGFC (t-PDGFC) (Figure 2). The truncated PDGFC isoform is generated due to retention of a 61 base pair exon resulting in a shift in the open reading frame, creating a stop codon. Interestingly, an alternative start codon in-frame at methionine[164] generates an N-terminally truncated isoform of PDGFC. This PDGFC mRNA splice variant has been demonstrated in fetal brain (22) and in breast cancer cell lines (our laboratory's unpublished data). Additionally, our laboratory screened a panel of breast carcinoma cell lines and found that there was no correlation between the malignant potential of breast carcinoma cell lines and the expression levels of PDGF A, B, and D. However, we found increased PDGFC mRNA expression in breast cancer cell lines with high tumorigenicity and invasive potential. This suggests that PDGFC may be the relevant ligand for aberrant PDGF signaling in breast cancer.

Body

During the past funding period I examined the role of PDGFC and its splice variant in human breast cancer. To this end we had previously established three sets of breast cancer cell models: Model 1, MCF-7 breast cancer cells which normally express little endogenous PDGFC were transfected with control vector or FL-PDGFC (His-tagged) to overexpress FL-PDGFC. Model 2, MDA-MB-231 breast cancer cells which normally express high levels of both FL-PDGFC and t-PDGFC mRNA were transfected with control shScramble or PDGFC shRNA to knock down expression. Model 3, BT-549 breast cancer cells that express mostly endogenous FL-PDGFC were transfected with control vector or t-PDGFC (c-myc tagged). As shown in Figure 3A, the transfected cell lines express appropriately at the RNA level. In addition, note that the BT-549 cell line shows high levels of the PDGFR-α receptor at the RNA level (Figure 3A). PDGFC in the conditioned medium of the transfected cell lines shows predominantly the proteolytically processed form of FL-PDGFC (~18 kDa) by western blot (Figure 3B, arrow). As expected knocking down the expression of PDGFC with shRNA in the MDA-MD-231 shPDGFC shows significantly lower, almost undetectable levels of PDGFC in the concentrated conditioned medium as compared to the control transfected (shScram, Figure 3B). Interestingly, BT-549 cells transfected with t-PDGFC/myc show slightly higher levels of the proteolytically processed PDGFC in the concentrated conditioned medium (Figure 3B). It should be noted that BT-549 cells have high levels of PDGFR-alpha at the RNA level (Figure 3A), we believe the levels of PDGFC in the conditioned medium is lower despite high PDGFC RNA levels in BT-549 cells due to autocrine signaling via PDGFR-alpha receptor. Further evidence of autocrine signaling is demonstrated by higher levels of phospho-Akt and phospho-Erk in BT-549 t-PDGFC/myc transfected cells as compared to the control transfected cells (Figure 3C).
In addition, knockdown of PDGFC in MDA-MB-231 cells decreases anchorage independent growth as seen with soft-agar colony formation (Figure 4A, 4B). Furthermore, PDGFC knockdown decreases cellular proliferation both under normal serum containing conditions and under low serum conditions (Figure 4C, 4D).

Thus far in this study, cellular localization studies of PDGFC in human breast cancer cell lines have yielded very interesting results. We have localized PDGFC to not only the conditioned medium as would be expected of a known secreted growth factor, but also the nuclear fraction of the MCF-7 breast cancer cell lines transfected with FL-PDGFC (Figure 5). Upon subcellular fractionation PDGFC is found in the chromatin bound nuclear fraction of T47D parental cells which naturally express predominantly FL-PDGFC (Figure 6, Lane 4). This nuclear localization suggests a unique function for PDGFC intracellularly. Furthermore, after analyzing multiple breast cancer cell lines, nuclear PDGFC is in the proteolytically processed form (~18 kDa) in BT-549 cells (data not shown) and T47D cells (Figure 6), while in transfected MCF-7 cells, nuclear PDGFC is its full length unprocessed form (~50 kDa, Figure 5). This difference in the proteolytic processing of PDGFC in cell lines is possibly due to differing levels of protease activity amongst the different breast cancer cell lines. Treating T47D cells with a protease inhibitor cocktail to attempt to inhibit the processing of nuclear PDGFC has not yielded conclusive results, however future plans are to try different protease inhibitors and combinations of in order to try to discern which class(es) of protease are processing nuclear PDGFC. In addition, by utilizing protease inhibitors which are cell permeable or cell impermeable, we will be able to discern valuable information about if nuclear PDGFC is proteolytically processed extracellularly or intracellularly.

The cellular localization of the splice variant form of PDGFC, called t-PDGFC, has proven difficult with our current myc-tagged construct as seen by the lack of a distict band of the expected size between the 25 kDa and 37 kDa markers (Figure 5 Lanes 2). Furthermore, immunoprecipitation with c-myc antibodies has not yielded conclusive results (data not shown). Thus, future plans are to subclone the open reading frame of t-PDGFC into a His-tagged mammalian expression vector since His-tag previously has worked well for localization of full length PDGFC by western blot or immunoprecipitation in our laboratory.

Key Research Accomplishments

- Expression of t-PDGFC appears to increase secretion of the proteolytically processed form of FL-PDGFC in BT-549 cells and increases autocrine signaling as evidenced by the increase the downstream signaling of PDGFC through phospho-Akt and phospho-Erk.

- Knockdown of PDGFC expression decreases anchorage-independent growth and cellular proliferation.

- PDGFC is not only secreted extracellularly, but it also is localized to the nucleus, and more specifically it can be found in the chromatin bound nuclear fraction in breast cancer cell lines.

- In T47D and BT-549 breast cancer cell lines nuclear PDGFC is in the proteolytically processed form, while in transfected MCF-7 cell lines nuclear PDGFC is in its full length unprocessed form.

- Proteolytic processing of PDGFC may be done by multiple classes of proteases.

Reportable Outcomes

Wayne State University School of Medicine Graduate Student Research Day 2011 Poster Presentation "Subcellular Localization of PDGFC in Breast Cancer" September 29, 2011
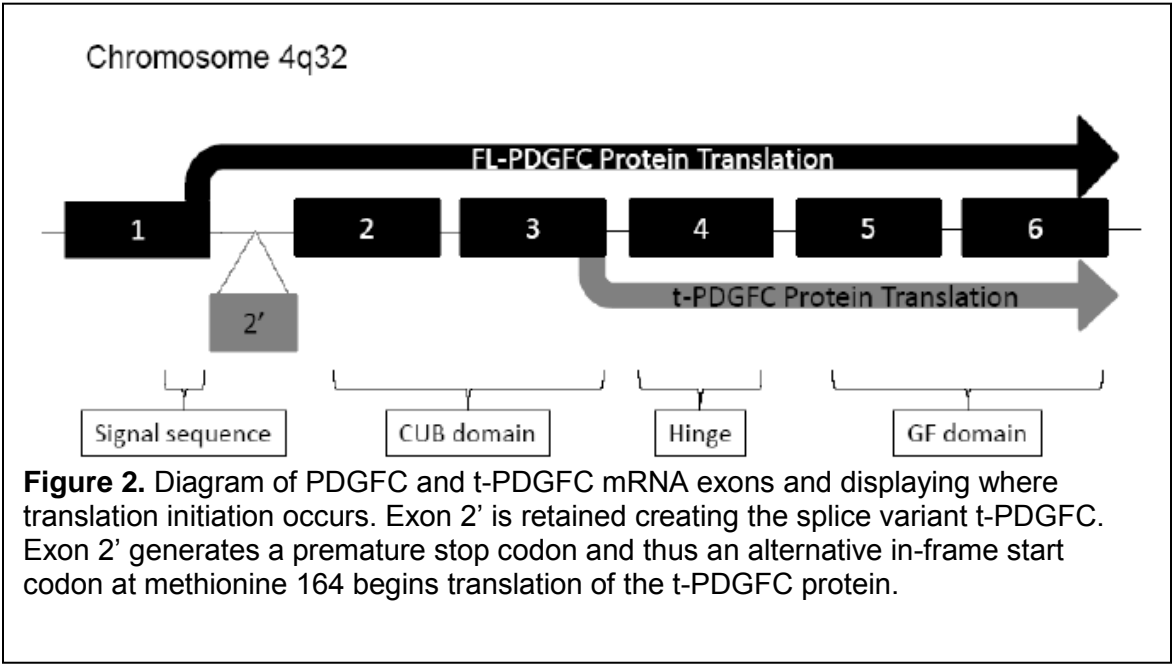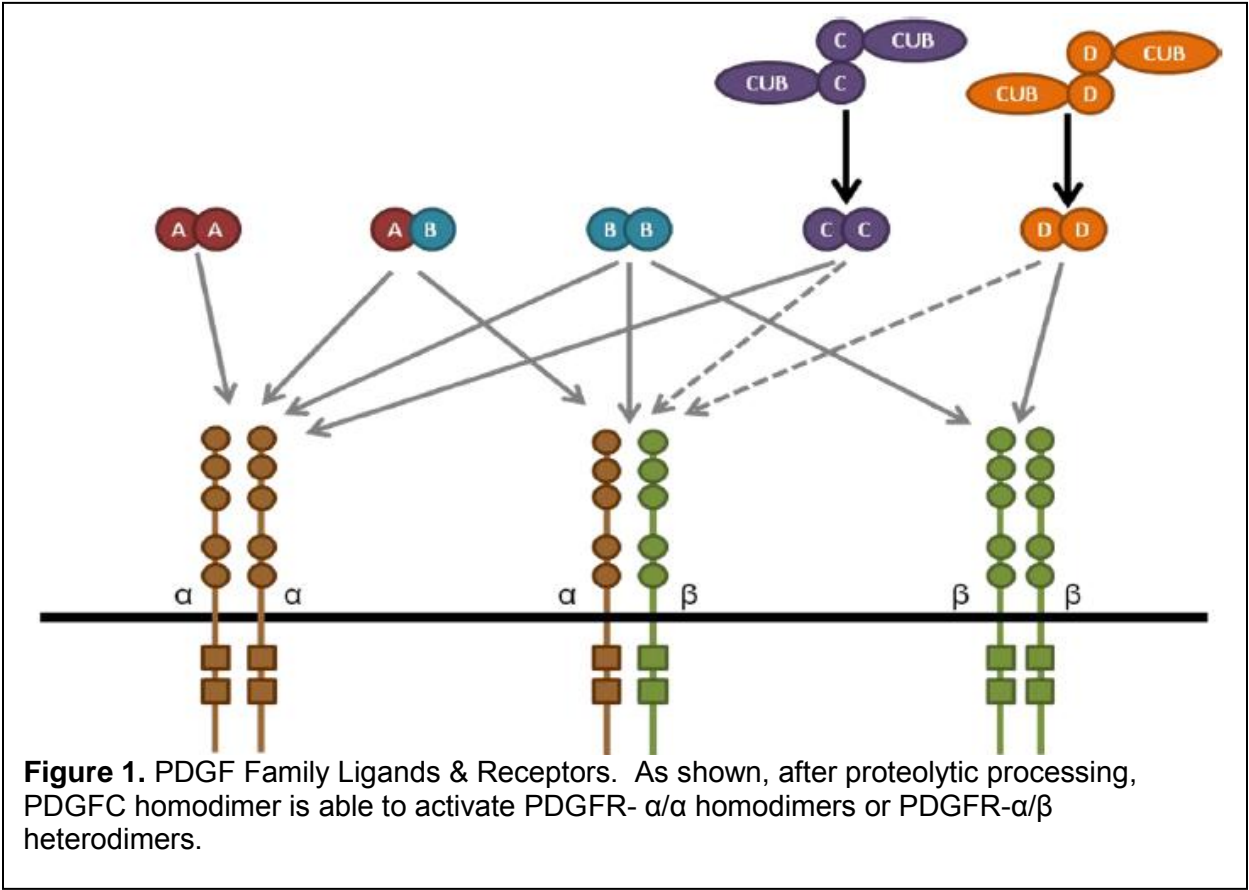
Wayne State University School of Medicine Department of Pathology Student Seminar Presentation "The role of PDGFC and its splice variant in breast cancer" March 2, 2011

Conclusion

The paradigm of growth factors in cancer is extracellular activation of a receptor. Currently there are several drugs approved by the United States Food and Drug Administration which target tyrosine kinases including the PDGF receptors. Imatinib mesylate (Gleevec®, STI571, Novartis) is the most notable of these tyrosine kinase inhibitors. In this study, utilizing subcellular localization, we show that PDGFC appears to not only be secreted into the extracellular space able to activate receptors on cells via paracrine & autocrine signaling, but we also show that PDGFC can be found in the nucleus of breast cancer cell lines. Nuclear PDGFC was reported in the literature in 2006 when Reigstad, et al described nuclear localization of full-length PDGFC in human papillary thyroid carcinoma samples and normal thyroid tissue samples from 14 patients by immunohistochemical (IHC) staining (*23*). The exact role of nuclear PDGFC is unknown; however, this demonstrates an important shift from the traditional model of growth factors as extracellular effectors to the novel roles of growth factors intracellularly. This has important implications for the treatment of breast cancer where blocking the effects of aberrant expression of growth factors in cancer progression may not be as simple as blocking their actions extracellularly at the receptor. Intracellular PDGFC may play an important role in breast cancer development or progression. Nuclear localization of growth factors could have important implications in how we understand and target cancer.

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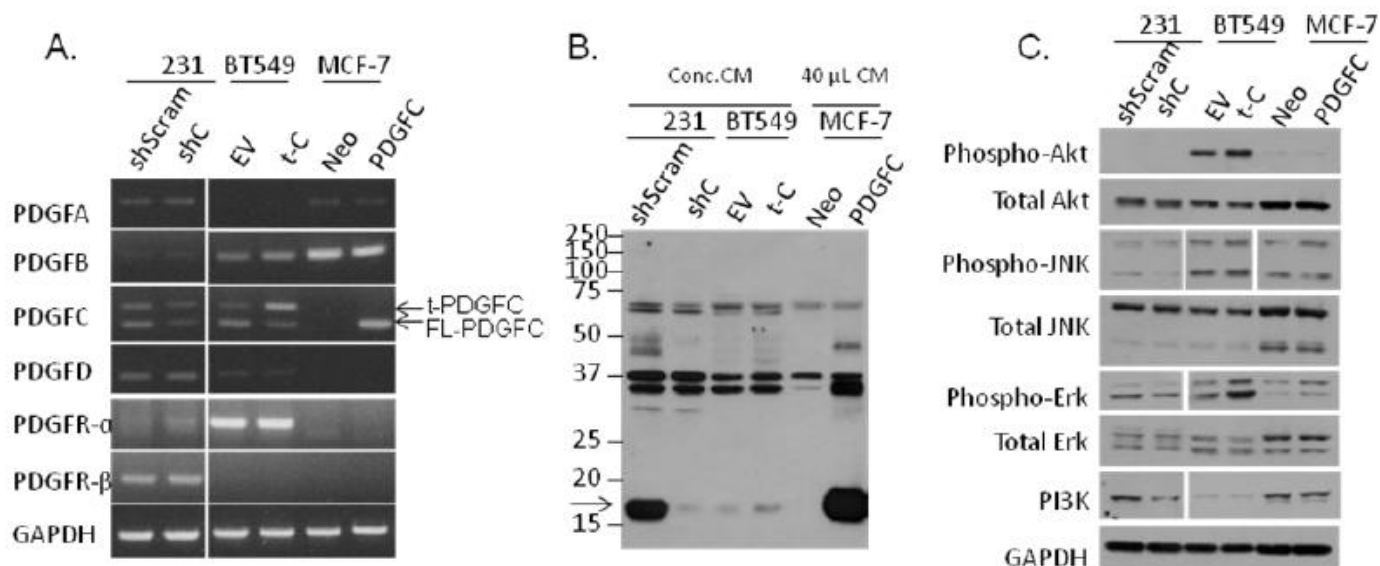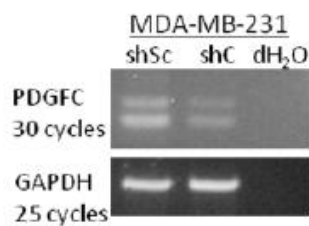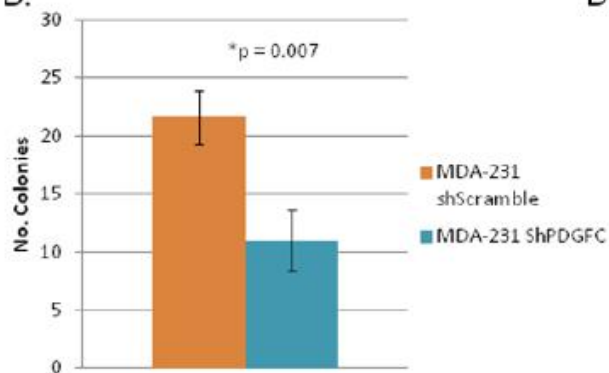
Figure 1. PDGF Family Ligands & Receptors. As shown, after proteolytic processing, PDGFC homodimer is able to activate PDGFR- α/α homodimers or PDGFR-α/β heterodimers.

Figure 2. Diagram of PDGFC and t-PDGFC mRNA exons and displaying where translation initiation occurs. Exon 2' is retained creating the splice variant t-PDGFC. Exon 2' generates a premature stop codon and thus an alternative in-frame start codon at methionine 164 begins translation of the t-PDGFC protein.

Figure 3. A. PCR of the transfected breast cancer cell models used in this study. **B.** Western blot of conditioned medium from the transfected breast cancer cell models probed for PDGFC. **C.** Western blot of whole cell lysate from the transfected breast cancer cell models. 231 shScram = MDA-MB-231 shScramble control transfected. 231 shC = MDA-MB-231 shPDGFC transfected. BT-549 EV = BT-549 empty vector control transfected. BT-549 t-C = BT-549 t-PDGFC/myc transfected. MCF-7 Neo = MCF-7 Neo control transfected. MCF-7 PDGFC = MCF-7 PDGFC/His transfected.
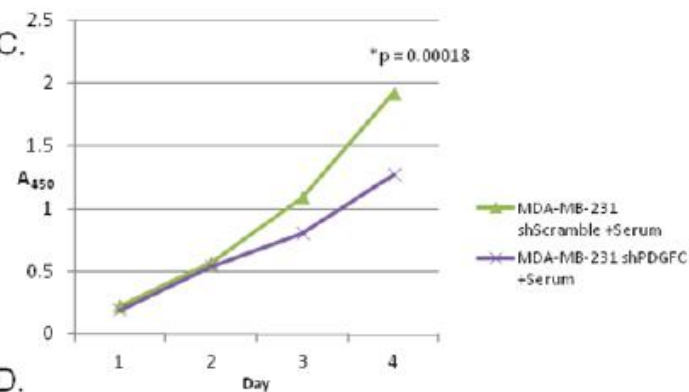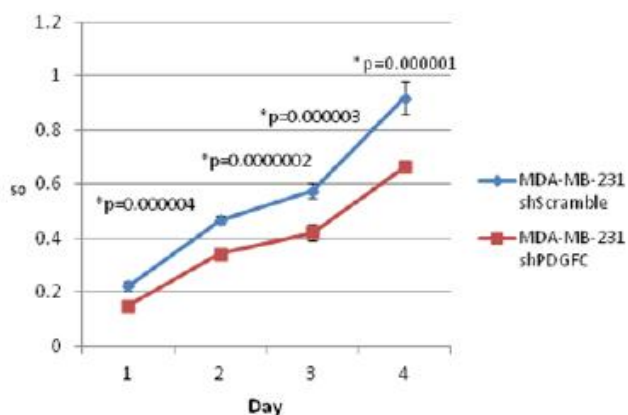
Figure 4. A. PCR of MDA-MB-231 cells transfected with control (shScramble, shSc) or PDGFC (shPDGFC, shC) shRNA. **B.** Soft Agar Colony formation **C.** WST-1 cell proliferation assay under normal serum containing conditions **D.** WST-1 cell proliferation assay under low serum conditions.
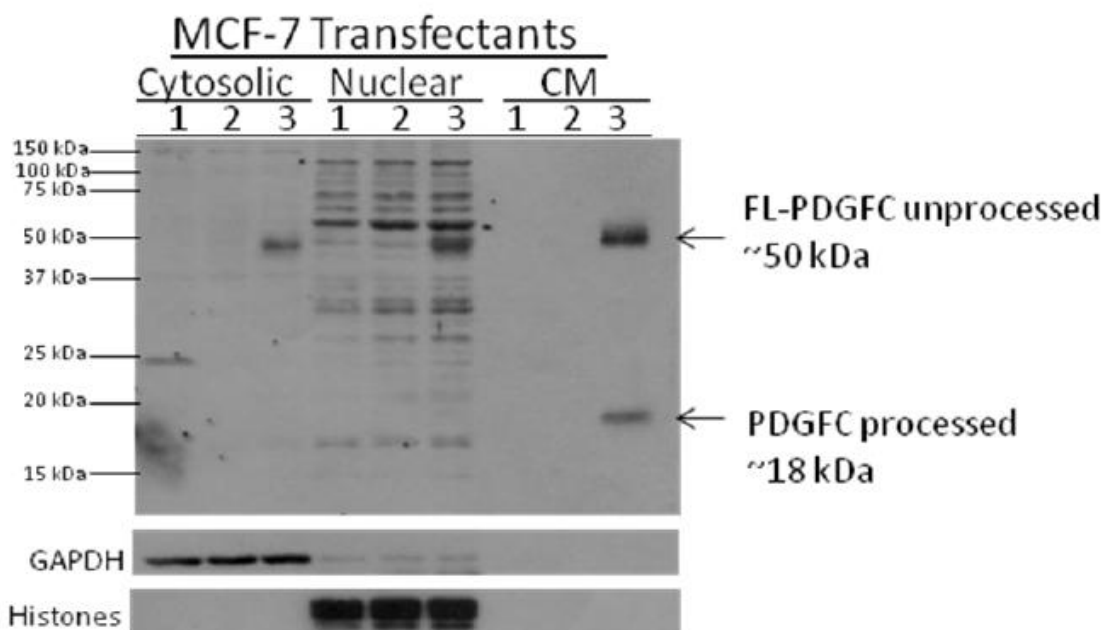
Figure 5. MCF-7 transfectants cellular fractionation western blot probed for PDGFC. **Lanes 1)** MCF-7 Empty Vector control transfected. **Lanes 2)** MCF-7 t-PDGFC/Myc transfected. **Lanes 3)** MCF-7 FL-PDGFC/Myc transfected. GAPDH is used as a marker for the cytosolic fraction and histones are used as a marker for the nuclear fraction. FL-PDGFC unprocessed is seen in all three fractions, while the processed form of PDGFC is seen only in the conditioned medium of MCF-7 cells transfected to express FL-PDGFC/Myc. Cytosolic = cytosolic fraction. Nuclear = nuclear fraction. CM = conditioned medium.
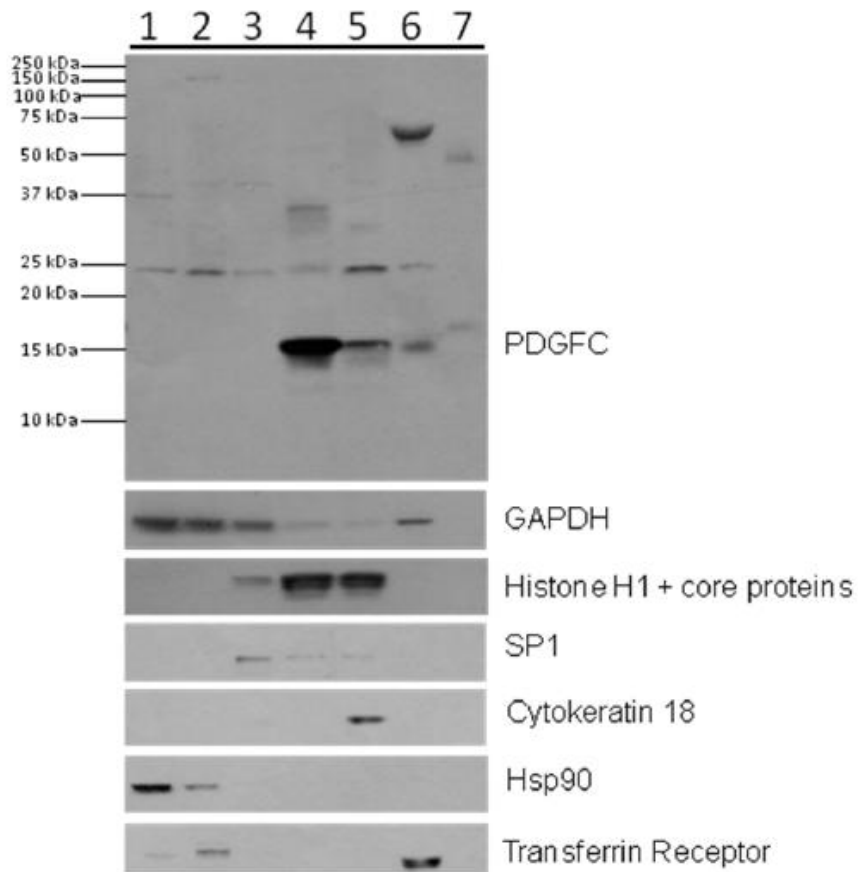
Figure 6. Immunoblot subcellular fraction of T47D parental cells.
Lane 1) Cytoplasmic fraction. **Lane 2)** Membrane fraction. **Lane 3)**
Soluble nuclear fraction. **Lane 4)** Chromatin bound nuclear fraction.
Lane 5) Cytoskeletal fraction. **Lane 6)** Concentrated conditioned
medium. **Lane 7)** Positive control PDGFC/His-tag conditioned
medium. PDGFC/His (Lane 7) migrates ~18 kDa, while endogenous
PDGFC of T47D parental cells (Lanes 4 – 6) migrates at a slightly
smaller molecular weight. Markers of different fractions are shown:
GAPDH & Hsp90 predominantly in the cytoplasmic fraction, Histones
predominantly in the chromatin bound fraction, and SP1
predominantly in the soluble nuclear fraction, cytokeratin 18
predominantly in the cytoskeletal fraction, and transferrin receptor as
a marker of the membrane fraction.